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Effects of L-lactic acid and D,L-lactic acid on viability and osteogenic differentiation of mesenchymal stem cells

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Poly(lactic acid) (PLA) and other aliphatic polyesters containing the unit of lactic acid are very popular biodegradable materials. While the degradation products, lactic acids, have been worried to bring with negative influence on biocompatibility, the focused experimental studies are less reported. This study is aimed at an *in vitro* examination of cytotoxicity of both L-lactic acid and D,L-lactic acid. Mesenchymal stem cells (MSCs) derived from rat bone marrow are employed to test the cytotoxicity of the lactic acids. Considering that the addition of lactic acids not only introduces lactate groups but also alters medium pH and ion strength, these three candidate effects are examined in a decoupled way by setting different comparison groups. The results confirm that the change of medium pH is the predominant factor. It has also been found that D-lactate is more cytotoxic than L-lactate at high concentrations. Yet, either L- or D,L-lactic acids seem acceptable in most of medical applications, because the cytotoxicity is significant only when the concentrations are as high as 20 mmol/L for both of them.

biodegradable polymer, biomaterial, tissue engineering, lactic acid, cytotoxicity, biocompatibility, mesenchymal stem cells

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Biodegradable polyesters especially poly(L-lactic acid) (PLA), poly(D,L-lactic acid) (PDLLA), and poly(D,L-lactic acid-co-glycolic acid) (PLGA) are widely used in biomedical fields [1–5]. These materials exhibit advantages such as good processibility [6–9] and tunable degradability [9–13]. That is why polyesters have been studied for a long time [14–20]. A lot of efforts have been done on improving the biocompatibility of the polyester porous scaffolds [21–28], and examinations of their medical applications [27–33].

Both *in vitro* and *in vivo* degradation behaviors of polyesters have been investigated [34–38]. Polyesters degrade via bulk hydrolysis of ester bonds. The acidic degradation products can be removed by the milieu solution or body fluids, and can be metabolized by organs such as liver and kidney. Nevertheless, polyesters are criticized due to their acidic degradation products [39,40]. If the degraded acids cannot be refreshed effectively, the acid may be accumulated; some reports about the failure of restoration by polyester implants [41,42] did draw attention of researchers.

There are two main reasons of the adverse effects, aseptic inflammation in host response or direct cytotoxicity of the degradation products. The present manuscript is only about the direct cytotoxicity. While the negative effects of the acidic degradation products such as lactic acids on cells are worried frequently, the focused investigations are rather limited. It is thus meaningful for an objective evaluation of the effects of lactic acids on cell behaviors.

The local accumulation of lactic acids must lead to a medium pH change, a lactate ion increase and an ion strength raise. How to decouple these three factors and determine the main one is an important topic, although some single factors have been examined [43,44]. Another fundamental question is the possible difference of cell responses between the two optical isomers of lactic acids. It is known that L-lactic acid occurs much more frequently than D-lactic acid. So, D-lactic acid might be harmful compared to more "natural"

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L-lactic acid or might not be. The answer depends upon experimental evidence, which is, however, never been afforded so far, to the best of our knowledge. This paper will address this question and give a preliminary answer.

In this paper, mesenchymal stem cells (MSCs) from bone marrows of rat are employed as the cell model, and the *in vitro* effect of the two isomers of lactic acids on cell behaviors is examined, as schematically presented in Figure 1. Both cell viability and osteogenic differentiation are detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and alkaline phosphatase (ALP) staining, respectively. A series of experimental groups is designed to decouple the three factors upon addition of lactic acids, namely, changes of medium pH, ion strength as well as a new chemical of lactate.

While both L-lactic and D,L-lactic acids are quite popular, pure D-lactic acids are rarely used, and much more expensive than either L-lactic acid or D,L-lactic acid. So, L- and D,L-lactic acids are compared in this study. Besides, in order to determine the critical concentration of lactic acid inducing cytotoxicity, whether or not there is any difference of the effects between L-lactic acid and D,L-lactic acid is tried to elucidate for the first time.

1 Materials and methods

1.1 Materials

L-lactic acid and D,L-lactic acid were purchased from Acros Organics. Sodium L-lactate, sodium D-lactate, MTT, ascorbic acid-2-phosphate, β-glycerophosphate, dexamethasone, leukocyte ALP kit (with naphthol AS-MX phosphate, citrate concentrate and fast blue RR salt included), and TRIzol reagent were obtained from Sigma-Aldrich. Low-glucose Dulbecco's modified Eagle's medium (DMEM), high-glucose DMEM, fetal bovine serum (FBS), Trypsin-EDTA and phosphate buffer solution (PBS) were obtained from Gibco. LIVE/DEAD® Viability/Cytotoxicity Kit was purchased from Molecular Probes. PrimeScript® RT reagent Kit with gDNA Eraser was bought from TaKaRa. Rotor-Gene™ SYBR® Green PCR (polymerase chain reaction) kit was offered by Qiagen. Milli-Q water was used in the experiments. All the other chemicals were of analytical grade and used without further treatment.

1.2 Isolation of MSCs

MSCs were isolated from bone marrow of 7-d new-borne Sprague-Dawley (SD) rats and cultured in low-glucose DMEM supplemented with 10% FBS in the incubator at 37°C with 5% CO_2 atmosphere and 95% humidity. Upon reaching about 90% confluence, MSCs were detached with Trypsin-EDTA and delivered to the next passage. The MSCs of the second passage were used in later experiments.

1.3 Setting of examined groups

The effects of lactic acids on cell behaviors were tested through adding lactic acids into the culture medium. A series of experimental groups as listed in Table 1 were designed to decouple the three candidate factors, medium pH, ion strength and lactate. Considering the costs, just L- and D,L-lactic acids will be compared in this study. For lactate sodium, we will simply use D- and L- one, and both of them are not very expensive.

1.4 Culture and osteogenic induction of MSCs

Cells were seeded at the density of 5000 cells/cm^2. The culture medium was 90% low-glucose DMEM with 10% FBS. The osteogenic induction medium was composed of 90% high-glucose DMEM, 10% FBS, 50 μmol/L ascorbic acid-2-phosphate, 10 mmol/L β-glycerophosphate and 100 nmol/L dexamethasone. 6 h after seeding, the culture medium was removed and replaced by the fresh medium supplemented of different additives in the corresponding groups, as shown in Table 1. The control group was a normal medium without extra additive. The culture media were replaced every 3 d.

1.5 Live/Dead assay

MSCs were seeded in 12-well tissue culture plates (Costar, Corning). After 3 d of culture, the cells were stained with the Live/Dead kit according to the manufacturer's protocol.

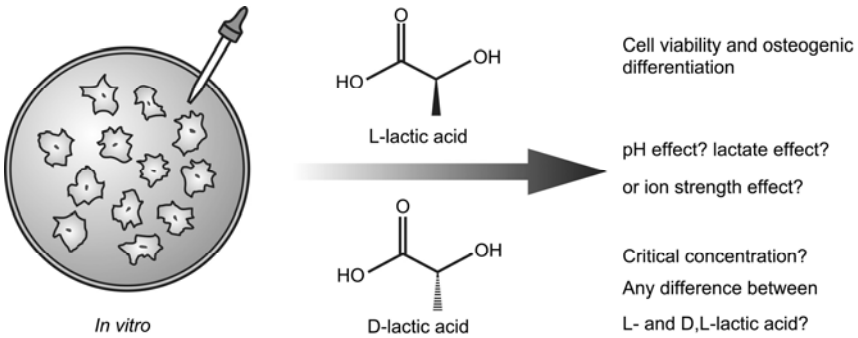

Figure 1 Schematic graph of our studies of effects of L- and D,L-lactic acid on viability and differentiation of MSCs.

Table 1 Examined groups with indicated additives in cell culture medium[a)]

Group[b)]	Additive[c)]
A (LLA)	L-lactic acid
B (DLLA)	D,L-lactic acid
C (HCl)	Hydrochloric acid
D (NaOH)	Sodium hydroxide
E (NaLLA)	Sodium L-lactate
F (NaDLA)	Sodium D-lactate
G (NaCl)	Sodium chloride
Control	None

a) The cell culture medium was DMEM supplemented with 10% FBS. The high-glucose DMEM was used in osteogenic induction, and all the other cases referred to low-glucose DMEM. b) Comparison between groups A and B revealed the difference between L- and D,L- lactic acids; groups C and D versus groups A and B revealed the underlying pH effect; groups E and F versus groups A and B revealed the underlying effect of lactate group; groups G versus groups A and B revealed the underlying effect of ion strength. c) Four concentrations, 5, 10, 20 and 40 mmol/L were examined in each group. The comparison among them gave the critical cytotoxicity concentration.

Briefly, just before staining, calcein AM and ethidium homodimer-1 were diluted in PBS. Afterwards, the culture medium was replaced by the staining solution and incubated at 37°C for 30 min, and finally, the cells were observed in an inverted fluorescence microscope (Axiovert 200, Zeiss).

1.6 MTT assay

MSCs were seeded in 96-well tissue culture plates (Costar, Corning). After culture for 1 and 3 d, an MTT solution (5 mg/mL in PBS) was added into the culture medium (volume ratio 1∶10); then the cells were incubated for another 4 h. MTT (yellow) was converted into a water-insoluble formazan salt (purple) by the metabolic activity of viable cells. After removing the medium, dimethyl sulfoxide (DMSO) was added to dissolve the formazan salt. Then the absorbance or optical density (OD) of the purple formazan solution was detected at 492 nm in a Multiskan instrument (Thermo Labsystems).

1.7 Osteogenic induction of MSCs, ALP staining and quantification

MSCs were seeded in 12-well tissue culture plates. After culture for 6 h, the cells were induced in the osteogenic medium for 7 d. Cells were then stained with leukocyte ALP kit according to the manufacturer's instruction. Briefly, cells were fixed with acetone/citrate, washed with Milli-Q water, and stained with Fast Blue RR/naphthol. The stained cells were observed in an inverted fluorescence microscope in the mode of bright field.

After that, the blue products were dissolved in tetrahydrofuran (THF), and the absorbance of the solutions at 570 nm was detected to quantify the ALP activity.

1.8 RNA extraction and real-time PCR

After 7 d of osteogenic induction of MSCs, the gene expression of ALP was detected by real-time PCR. The total cellular RNA was extracted by TRIzol reagent, which concentration and purity were measured by NanoDrop 2000 (Thermo Scientific). The 1st strand of complementary DNA (cDNA) was reversely transcribed from RNA using PrimeScript® RT reagent kit according to the manufacturer's protocol. Then real-time PCR quantitation of the mRNA was performed on Rotor-Gene™ Q 2plex (Qiagen) using Rotor-Gene™ SYBR® Green PCR kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was set as the housekeeping gene and used as an endogenous reference to normalize the calculation based on the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 2.

1.9 Statistical analysis

For all experiments, data were reported as mean plus standard deviation. The experiments were conducted at least twice to ensure reproducibility. Student's *t*-test was employed, and a statistical significance was set at $P<0.05$.

2 Results

2.1 pH values of cell culture media with additives

The pH values of culture medium in different study groups were quantified by a digital pH meter. The results of both low-glucose DMEM and high-glucose DMEM are shown in Figure 2. The presence of glucose did not influence medium pH.

2.2 Cell viability

Using Live/Dead kit, live cells were stained by calcein AM, producing bright green fluorescence, and dead cells were stained by ethidium homodimer-1, producing red fluorescence. The results are shown in Figure 3. Below 20 mmol/L of those additives, cells were well alive in all the groups, and

Table 2 Primers used for real-time PCR

Target gene	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
ALP	ATGGTAACGGGCCTGGCTACA	AGTTCTGCTCATGGACGCCGT
GAPDH	GCTCTCTGCTCCTCCCTGTTCTAG	TGGTAACCAGGCGTCCGAT

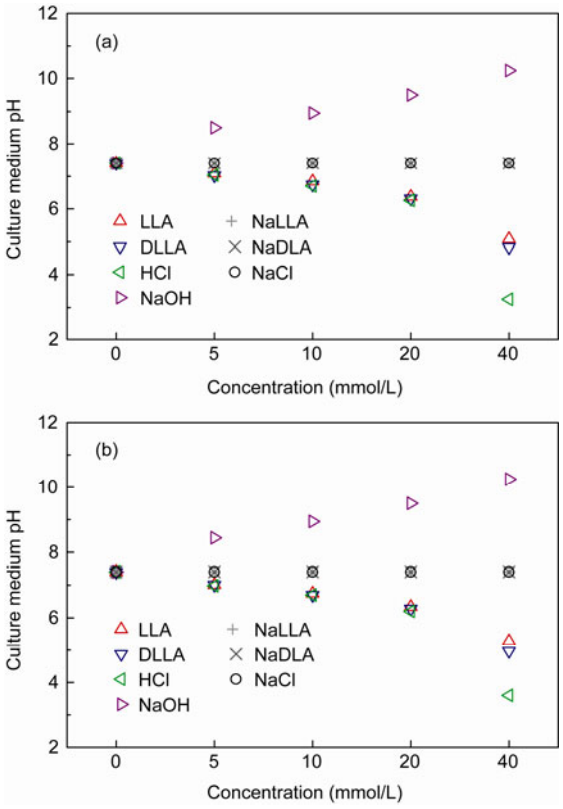

Figure 2 pH values of (a) low-glucose DMEM (culture medium) and (b) high-glucose DMEM (induction medium) in the indicated experimental groups.

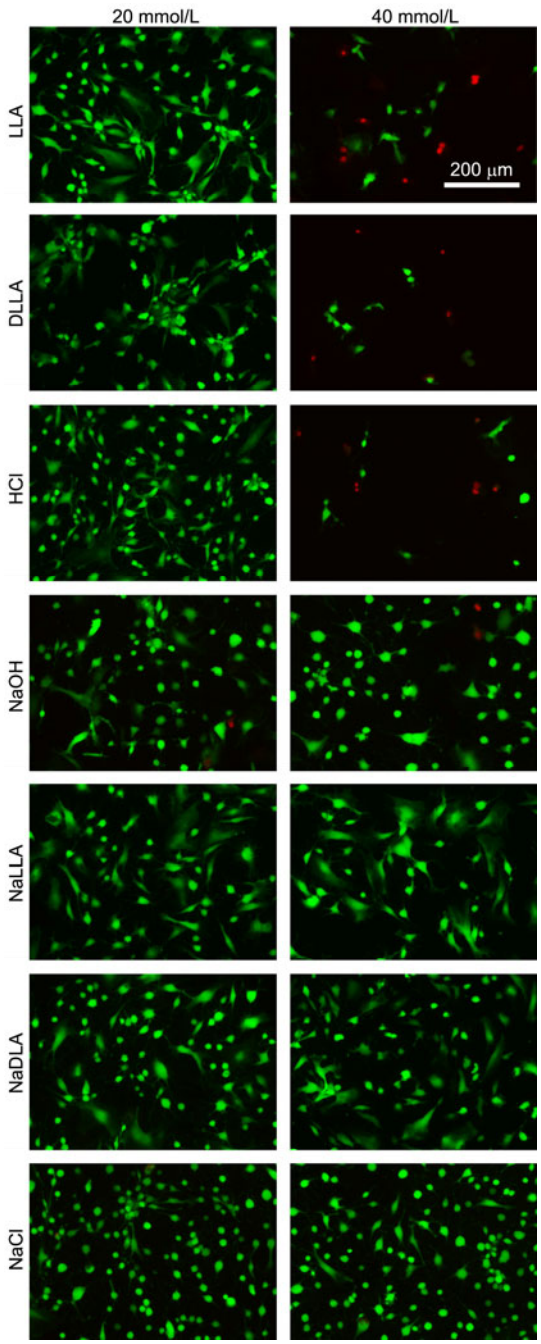

Figure 3 Fluorescent micrographs of MSCs cultured for 3 d and then stained with Live/Dead assay in the indicated groups. Green and red fluorescences represent live and dead cells, respectively.

few dead cells were observed. At 40 mmol/L, the live cell numbers dropped and dead cell numbers increased in the groups LLA, DLA and HCl; a large amount of live cells and only a few dead cells could be observed in group NaOH; seldom dead cells were seen in the groups NaLLA, NaDLA and NaCl.

The cell viabilities tested by MTT assay after culture of 1 and 3 d are presented in Figure 4. To evaluate the cytotoxicity grade, relative growth rate (RGR) is employed, which is defined as OD_{exp}/OD_{contr}. Here, OD_{exp} is the optical density detected from the experimental group, and OD_{contr} is that from the control group. According to the national standard GB/T 16886.5-2003 and international standard ISO 10993-5:2009(E), there are six cytotoxicity grades with 0, 1, 2, 3, 4 and 5 defined by RGR⩾100%, 75%–99%, 50%–74%, 25%–49%, 1%–24% and 0, respectively [45]. Grades 0 and 1 are supposed no cytotoxicity. The dashed lines in Figure 4 indicate RGR 75%. The groups of NaOH, NaLLA, NaDLA and NaCl did not exhibit significant cytotoxicity at all of the examined additive concentrations. For the groups of LLA, DLLA and HCl, cytotoxicity appeared at high concentrations.

2.3 Osteogenic differentiation

After osteogenic induction for 7 d, the ALP activity was visualized after staining cells by Fast Blue RR/naphthol. Some typical micrographs are shown in Figure 5. We also resolved the stained products and quantified the ALP activities, with the results presented in Figure 6. Compared to the groups of NaOH, NaLLA, NaDLA and NaCl, the groups of LLA, DLLA and HCl exhibited more significant inhibition of the ALP activity at high concentrations.

ALP gene expressions in some groups were further detected by real-time PCR. Figure 7 strengthens the lower

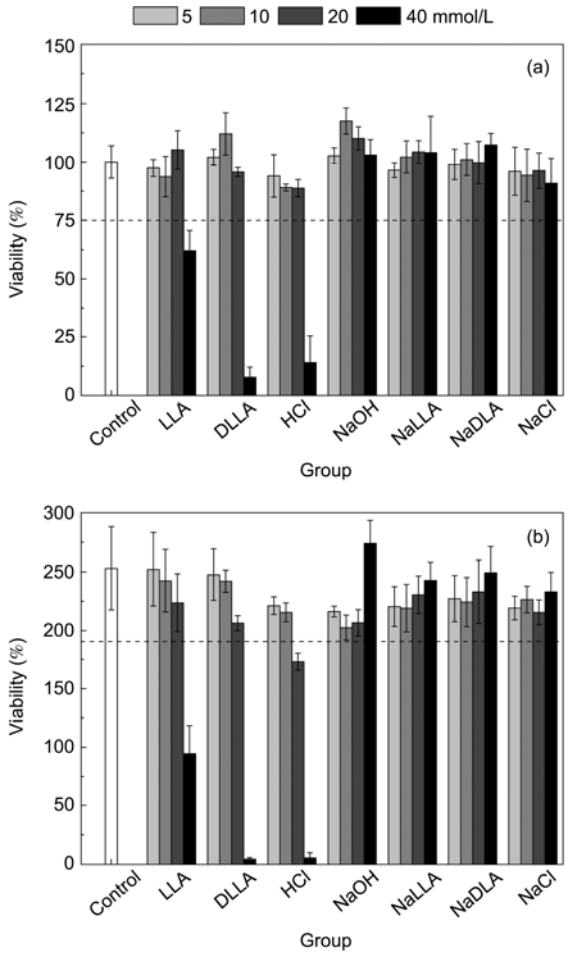

Figure 4 Viability of MSCs tested by MTT assay (n=4). Setting the optical density of control group after 1-d culture as 100% viability, the viabilities of all the other experimental groups after (a) 1- and (b) 3-d cultures were normalized. The dashed lines indicate relatively 75% viability, and the relative viability over 75% is supposed no cytotoxicity. t-Tests are made between experimental groups and the control group. The P values are listed in Tables S1–S4.

relative expression of ALP in groups DLLA and NaDLA than groups LLA and NaLLA, respectively.

3 Discussion

Biodegradable polyesters such as PLA and PLGA are commonly used in tissue engineering and regenerative medicine. While many investigations of the polymers and their degradation kinetics have been made, few studies are focused upon the degradation products. The degradation products are complicated, and composed of oligomers (intermediate products) and lactic acids (final products). The final products contribute to the acidity more due to much larger molar numbers, and the present paper is focused on the effects of lactic acids. Lactic acids have two different isomers with L- and D-configurations. MSCs derived from bone marrows are one of the most important cell sources used in tissue

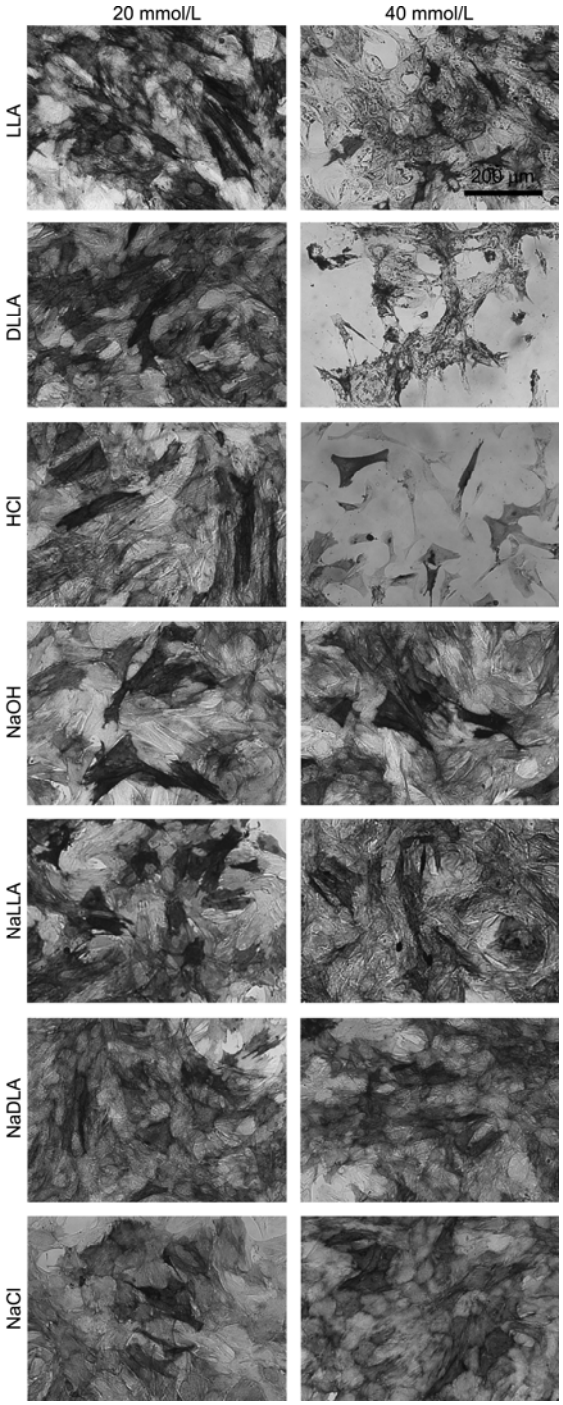

Figure 5 Bright-field micrographs of ALP-stained cells of indicated groups after 7 d of osteogenic induction of MSCs.

engineering [18,29]. Herein, we investigated the effects of L-lactic acid and D,L-lactic acid on viability and osteogenic differentiation of MSCs *in vitro*.

3.1 Which is the main factor underlying the lactic acid effect, medium pH, lactate group or ionic strength?

There are three candidate factors leading to the lactic acid

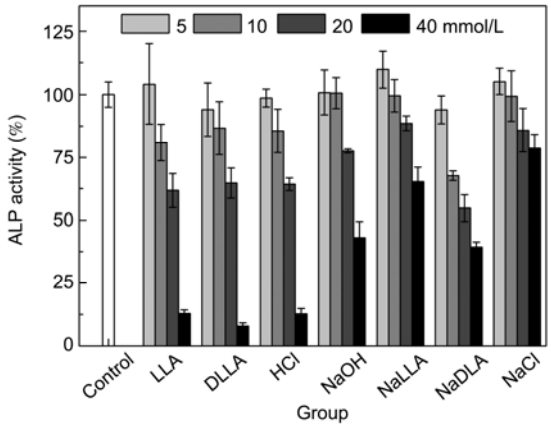

Figure 6 ALP activity of MSCs for 7 d of osteogenic differentiation induction in the indicated groups (n=4). The P values are listed in Tables S5–S7.

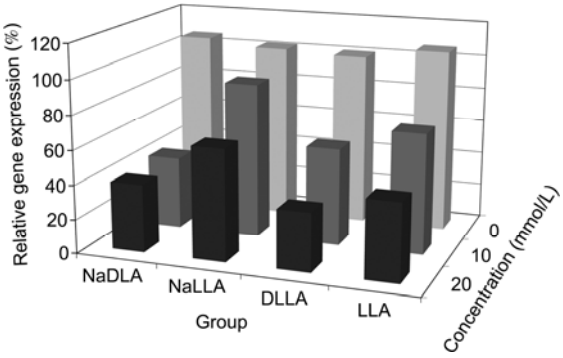

Figure 7 Gene expression of ALP of MSCs tested by real-time PCR on day 7 after culture in the osteogenic induction medium (n=3). GAPDH was set as the housekeeping gene. The P values are listed in Table S8.

effect, medium pH decrease, lactate group accumulation and ion strength increase, as schematically presented in Figure 1. So, we designed seven groups, as listed in Table 1, to decouple these factors. MSC viabilities under treatments of those additives are shown in Figure 4. Group NaCl was set to investigate the ion strength effect, and the result showed that MSC viability was not affected obviously; groups NaLLA and NaDLA were set to investigate the lactate effect, and MSC viability was not significantly affected either. So the ionic strength and lactate chemical were not the main factors to influence the MSC viability within the concentrations examined in this study. Medium pH did drop after addition of sufficient LLA, DLLA and HCl (Figure 2), and then significant cytotoxicity was found (Figures 3 and 4).

Besides MSC viability, osteogenic differentiation was also tested using ALP activity as the main indicator. Comparison between the seven groups in Figures 5 and 6 supports again that medium pH is the main underlying factor of lactic acids. Disthabanchong et al. [43] examined the *in vitro* osteogenic differentiation of human MSCs at different medium pH by addition of HCl (yet lack of the groups of lactic acids, sodium lactates, NaCl and NaOH), and our results of rat MSCs under various acidic pH are consistent with theirs. Our studies confirm the reasonability to prepare organic-inorganic composites to compensate pH by mixing some alkaline inorganics into polyester-containing polymers [9,21–24].

Our tests of addition of NaOH also indicated that a minor alkaline environment is beneficial for cell viability of MSCs (group D in Figure 4). It supports that some inorganic materials such as hydroxyapatite (HA) are good bone-repairing materials and helps to partially understand why HA has been widely existed in the native bone matrix.

3.2 Is there any significant difference of cytotoxicity between L- and D,L-lactic acid?

L-lactic acid is more "natural" in nature. In order to control the mechanical and degradation kinetics, polymers containing D,L-lactic acids are also quite commonly applied in biomedical applications. We are here curious about the basic question whether or not there is any difference of cytotoxicity between them. The MTT arrays in Figure 4 showed a significant difference between groups LLA and DLLA at 40 mmol/L, and thus DLLA seemed more cytotoxic than LLA at high concentrations.

Despite data fluctuation, both cell viability from MTT assay (Figure 4) and the ALP activity in the test of osteogenic differentiation (Figure 6) illustrate that LLA is better than DLLA especially at high concentrations. Nevertheless, such differences are not very significant at relatively low concentrations. To the best of our knowledge, besides L-lactate, D-lactate can also be involved in human and ruminant metabolism, but its metobolism rate is slower for the lack of D-lactate dehydrogenase *in vivo* [46]. A more interesting topic, although beyond the present manuscript, might be the *in vivo* examination of D-lactic acid versus L-lactic acid and of implanting materials containing D-lactic acid versus L-lactic acid. Hence, one does not need to overly worry the D-configuration, although this topic is worthy of more experimental examinations especially *in vivo* tests in the future.

3.3 What might be the tolerant concentration of lactic acid?

According to the standard (GB/T 16886.5-2003 and ISO 10993-5∶2009(E)), RGR above 75% (cytotoxicity grades 0 and 1) are supposed of no cytotoxicity [45]. Our tests indicate that the tolerant concentration of L-lactic acid and D,L-lactic acid for rat MSCs are between 20–40 mmol/L (Figure 4). Our observations are basically consistent with Chen et al. [47] in studies of lactic acid effects, although the D- and D,L-configurations have not been examined by them. No threshold of ALP activity to evaluate the side influence on osteogenic differentiation has been set so far. Yet, if we tentatively set 75% as the threshold, the tolerant concentrations for both L-lactic acid and L,D-lactic acid are between

10–20 mmol/L according to our examinations of rat MSCs, and additions of 40 mmol/L lactic acids resulted in decrease of ALP activity more than 50% relative to the blank control (Figure 6). In a previous study of growth inhibitors including lactic acids (lack of the groups of medium pH, salt, lactate, and D,L-lactic acids), Schop et al. [44] indicated the cell-type dependence of the response to L-lactic acids: rat MSCs was inhibited at 16 mmol/L and human MSCs at 35.4 mmol/L. So, human cells are more tolerant to lactic acids. Taking it into consideration and combination of our results of both cell viability (Figure 4) and ALP activity (Figure 6), we estimated 20 mmol/L as the critical cytotoxic concentration for both L-lactic acid and D,L-lactic acid.

Then, how about the accumulated lactic acid concentration or fluid pH after implanting an aliphatic polyester? The answer could be diverse, because pH is highly dependent upon the composition and amount of implanted materials, and also upon the fluid exchange rate at the implanting site. Some *in vivo* study cases [41,42] reported that massive acidic degradation products of bone screw or plate made of PLA or PLGA induced aseptic inflammatory reactions. But the negative results of those solid bone screws or plates cannot be simply extended to highly porous tissue engineering scaffolds, for the latter contains a much smaller amount of polyesters and much bigger surface areas for contacting with body fluid. Liu and Cao [39] pointed out that a subcutaneous implanting of polyesters can relatively easily trigger the host response but the implantation to other sites may not cause severe aseptic inflammatory reaction.

We think that it is not wise to stop further experiments of implanting at other sites simply based on a negative response in a pre-test of a subcutaneous implanting. Setting the joint site as an example, this site exhibits less host responses to implant, because more body fluids and faster fluid exchange are helpful for removing the acidic degradation products effectively. In fact, many positive results of tissue repairing using PLA or PLGA porous scaffolds have been reported [27–30,48,49]. Our group has recently revealed that the body fluid exchanged faster than we initially supposed, even at the subcutaneous site, for a block copolymer hydrogel composed of PLGA-PEG-PLGA with initial pH 4 became neutral just ten hours after a subcutaneous injection into SD rats [50]. So in the cases of implanting highly porous PLA or PLGA scaffolds in sites of sufficient fluid exchanges, the side effect of acidic degradation products of polyesters might be weakened, although further examinations both *in vitro* and *in vivo* of the cell or tissue responses are still called for.

4 Conclusions

We examined the *in vitro* effects of both L- and D,L-lactic acids on MSCs from bone marrow of rats through adding lactic acids into cell culture media. Seven groups were designed to decouple the factors of pH, lactate group, and ionic strength. Cell viability and osteogenic differentiation were detected to make comparison among those groups. The increase of the lactate group and the ionic strength did not play a critical role, and the main factor of lactic acids on cytotoxicity was confirmed to be the pH decrease. We also found that D-lactate was more cytotoxic than L-lactate at high concentrations. Yet the critical concentrations for both L- and D,L-lactic acid were as high as about 20 mmol/L. We deduce that one need not overly worry the acidic degradation products unless a large amount of aliphatic polyesters with a relative low porosity implanted at the site of a slow body fluid exchange rate.

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